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14. ABSTRACT
In many tumors, the actively dividing cells account for only a small proportion of the total, with the remainder of the cells being in a quiescent state or G0. Indeed, tumor cell population in G0 ranges from 70-95% and the quiescent cell population of tumors poses a barrier to the success of many cancer therapies. Numerous studies demonstrate that non-steroidal anti-inflammatory drugs (NSAIDs) hold significant promise as anti-cancer therapeutics.We started identifying now the best of NSAIDs therapy for apoptosis induction of quiescent ovarian cancer cells. In this report we show that structurally unrelated NSAIDS induces apoptosis in quiescent ovarian cancer cells. Strong inducers of apoptosis included flufenamic acid, flurbiprofen, celebrex and finasteride, whereas treatment with ibuprofen in low levels of apoptosis. Additionally, the spectrum of NSAIDs obtained in this analysis differs from the spectrum of NSAIDs obtained by our group for induction of apoptosis in proliferative ovarian cancer cells. In vivo experiments corroborated our in vitro data. The treatment with NSAIDs, which act in the proliferative stage in combination with NSAIDS, which act in the quiescent stage, had a synergistic effect in reducing tumor formation compared with single drug treatment. Our results offer a multitude of novel entry points for drug development and will provide opportunities for a rational design of new combination treatment modalities for ovarian cancer.

15. SUBJECT TERMS
quiescent stage; G0 stage; ovarian cancer, NSAIDs

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Introduction

In many tumors, the actively dividing cells account for only a small proportion of the total, with the remainder of the cells being in a quiescent state or G0. Indeed, tumor cell population in G0 ranges from 70-95%, among different types of cancer including epithelial ovarian cancer (EOC). The quiescent cells within the tumor are viable but in a reversible state of growth arrest. Nevertheless, the quiescent cell population of tumors poses a barrier to the success of many cancer therapies. Most chemotherapeutic drugs target proliferating cells, but the growth fraction of many tumors is low. Tumor cells in the quiescent stage are resistant to therapy and harbor the capacity to replenish a tumor after therapy. However, in cancers that poorly respond to therapy, it is the proliferating cells that are targeted. Therefore, it is tempting to explain all therapeutic failures by the persistence of tumor cells. Moreover, attempts to reduce G0 phase cells population in order to switch on cells into division cycle and progress to the chemosensitive phases have shown limited success. Numerous studies demonstrate that non-steroidal anti-inflammatory drugs (NSAIDs) hold significant promise as anti-cancer therapeutics. Our notion is that by identifying the best of NSAIDs therapy for apoptosis induction of quiescent ovarian cancer cells, we may be able to rationally design a combination of several drugs that with distinct target specificities that should act synergistically and, thus, more effectively against ovarian cancer. Our goal is to carry out a systematic survey of a panel of NSAIDs with regard to their ability to induce apoptosis in quiescent ovarian cancer. Our hypothesis is this analysis will offer a multitude of novel entry points for drug development and will provide opportunities for a rational design of new combination treatment modalities for ovarian cancer.

Body

Based on the approved Statement of Work the following research accomplishments are associated with each task outlined below:

Aim 1. Evaluate the efficacy of a whole set of NSAIDs to induce apoptosis in quiescent ovarian cancer cells. (1-6 months)

Multiple NSAIDs are potent inducers of apoptosis in G0 ovarian cancer cells. NSAIDs have been shown to elicit anti-cancer effects in various different settings; however, there has been very little effort to compare the effects of different NSAIDs to each other and to decipher the precise molecular mechanisms of action. We have started to systematically analyze the biological effects of a comprehensive set of NSAIDs in order to gain further insights into the molecular anti-cancer mechanisms of NSAIDs and to provide the basis for a rational application of NSAIDs as therapeutic agents for ovarian cancer. First, we used the starvation method for synchronize cells at specific quiescent G0 state which were analyzed by propidium iodide FACS analysis (see material and methods below). After checking that all cells were at GO stage, a broad panel of NSAIDs was tested for their abilities to induce apoptosis in cancer cells. The concentrations for all NSAIDs drugs used in this study were selected carefully and comparable to the achievable plasma concentrations (1-10). However, some drug concentrations exceeded thephysiologically achievable doses (1-10). Thus, a whole panel of NSAIDs including Aspirin, Ibuprofen, Exisulind, Acetaminophen, Naproxen, NS-398, Celecoxib, Diclofenac, Finasteride, Flufenamic acid, Meloxican, Ebselen and Flurbiprofen was tested for their abilities to induce

apoptosis in cancer cells. SW626, CAV, SKOV and 36M2 ovarian cancer cells (See Material and methods below). They were treated with 5mM Aspirin, 200μM Ibuprofen, 200μM Exisulind, 1mM Acetaminophen, 200μM Naproxen, 200μM NS-398, 50μM Celecoxib, 200μM Diclofenac, 50μM Finasteride, 200μM Flufenamic acid, 40μM Meloxican, 50μM Ebselen, 20nM Flurbiprofen or 50μM Sulindac sulfide. Apoptosis was measured 24 hours after treatment revealing that a variety of, but not all NSAIDs induced apoptosis in ovarian cancer cells. Strong inducers of apoptosis included Flufenamic Acid, Flurbiprofen, Finasteride, Celocoxib when compared with the solvent controls, whereas treatment with Ibuprofen resulted in low levels of apoptosis and the treatment with the remaining drugs did not result in apoptosis induction (Figure 1).

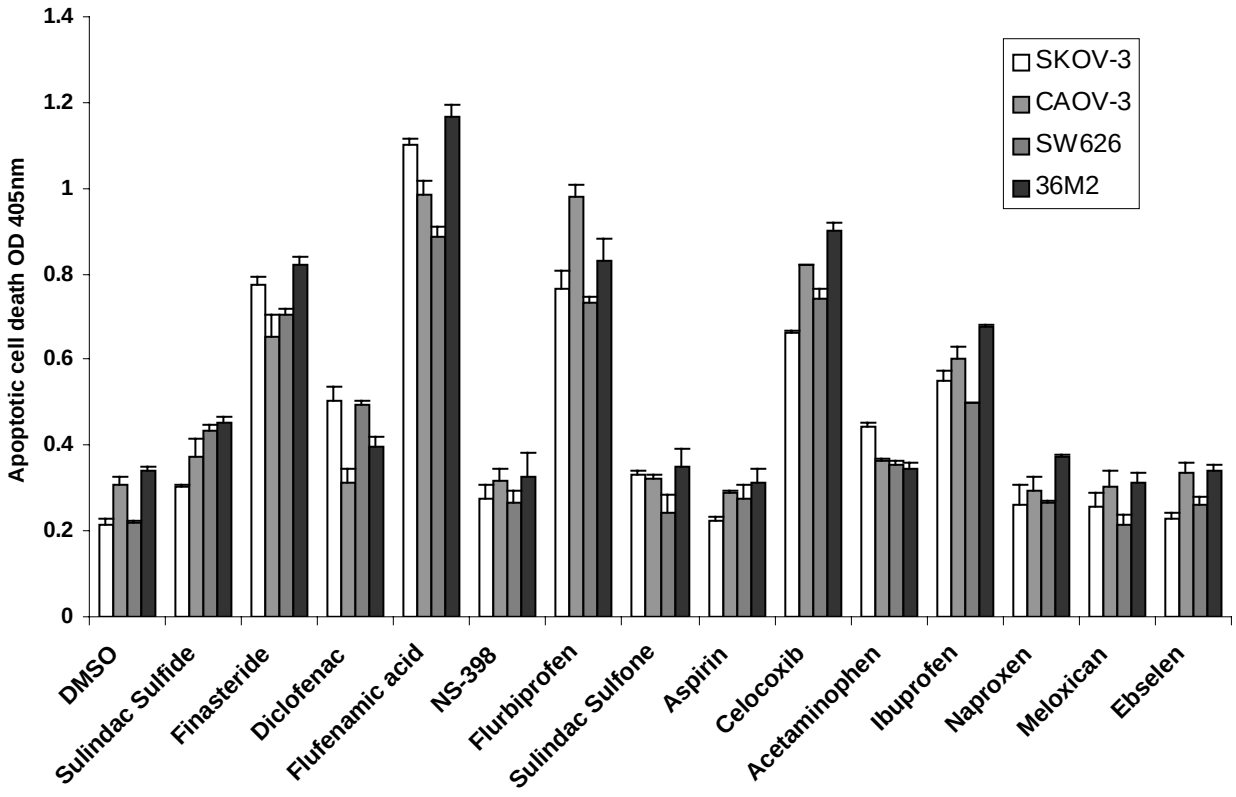

Figure 1. Multiple NSAIDs induce apoptosis in quiescent ovarian cancer cells. Apoptosis assay of SKOV-3, CAOV-3, SW626 and 32M2 quiescent ovarian cancer cells. After synchronizing the cells in G0 stage by starvation method, the cells were treated with 50 μM Sulindac sulfide, 5 mM aspirin, 200 μM ibuprofen, 200 μM Sulindac sulfone, 1 mM acetaminophen, 200 μM naproxen, 200 μM NS-398, 50 μM celecoxib, 200 μM diclofenac, 50 μM finasteride, 200 μM flufenamic acid, 40 μM Meloxican, 50 μM Ebselen, 20 nM Flurbiprofen and 50 μM Sulindac sulfide or DMSO and apoptosis were measured 24 hrs post-treatment. Data means ± s.d. of triplicate independent experiments for each treatment.

We extended our studies and determined the lowest dose of the stronger inducers, which still would have an effect in the programmed cell death of quiescent quiescent EOC cells. The concentrations of selected NSAIDs were chosen 2, 5 and 10 times lower than the physiologically achievable doses used in the experiments mentioned above. Apoptosis was measured in

quiescent EOC cells 24 hours after treatment with different doses of NSAIDs. Our results showed that the dose of Flufenamic acid can be reduced down 10 times, Flurbiprofen and

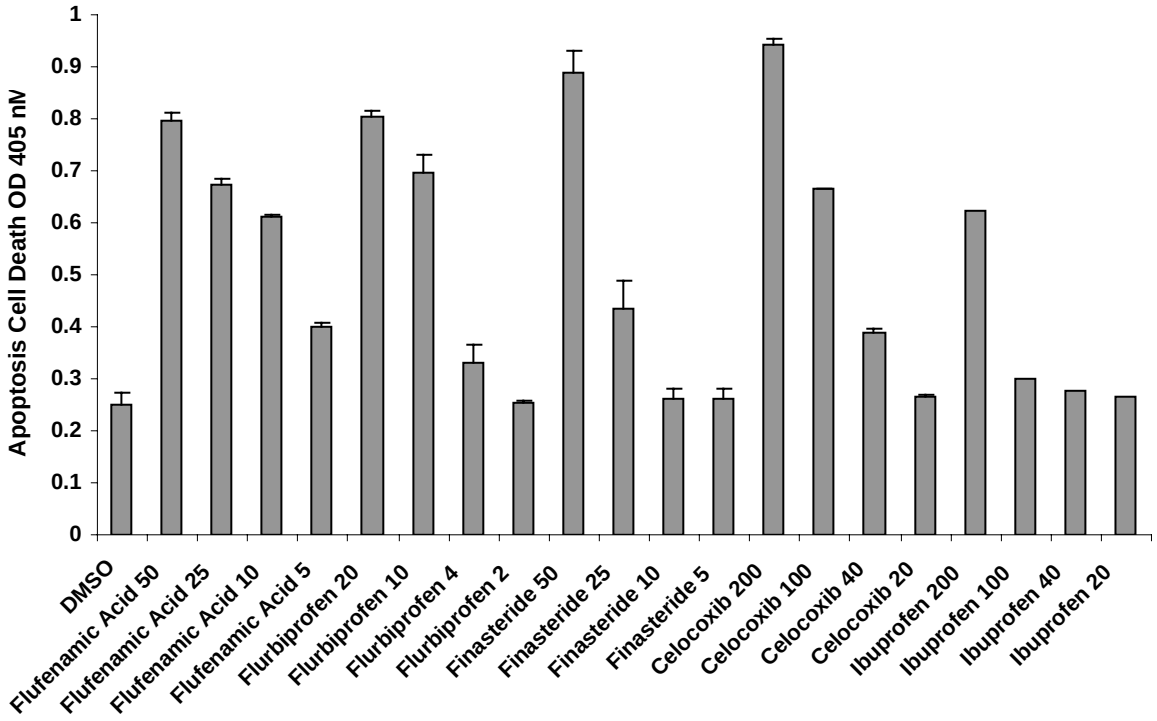

Figure 2. Multiple NSAIDs induce apoptosis in quiescent ovarian cancer cells. Apoptosis assay of SW626 quiescent ovarian cancer cells. After synchronizing the cells in G0 stage by starvation method, the cells were treated with 50, 25,10 and 5 μM Flufenamic acid; 20, 10, 4 and 2nM Flurbiprofen; 50, 25,10 and 5 μM Finasteride; 200, 100,40 and 20 μM Celocoxib; 200, 100,40 and 20 μM Ibuprofen or DMSO and apoptosis were measured 24 hrs post-treatment. Data means ± s.d. of triplicate independent experiments for each treatment.

Celocoxib can be reduced down 5 times whereas Finasteride can be reduced 2 times when compared with the solvent controls, and still resulting in apoptosis induction (Figure 2).

Methods:

Cell Culture: The ovarian cancer cancer cell lines SKOV-3, CAOV-3, M32 and SW626 were obtained from American Type Culture Collection, Rockville, MD and were grown in RPMI medium (GIBCO/BRL). The media were supplemented with 10% fetal bovine serum, 50U of penicillin per ml and 50 μg of streptomycin per ml (all from GIBCO/BRL). The cells were maintained in a 5% CO_2-humidified incubator at 37°C. For starvation method, the cells were cultivated in medias without fetal bovine serum for 24-48 hours.

Reagents: Sulindac sulfide, sulindac sulfone, ibuprofen, aspirin, acetaminophen, naproxen were obtained from Sigma-Aldrich (St. Louis, Missouri USA). Meloxican, celocoxib, diclofenac, finasteride and flufenamic acid were obtained from LKT laboratories (St. Paul, MN, USA). NS-398, ebselen and flurbiprofen were purchased from Calbiochem (San Diego, CA, USA). The drugs were dissolved in DMSO or ethanol. Cancer cells were treated in their particular medium

for 24h. The final concentration for each compound were: 50μM Sulindac sulfide, 5mM aspirin, 200μM ibuprofen, 50μM Sulindac sulfone, 1mM acetaminophen, 200μM naproxen, 200μM NS-398, 50μM celecoxib, 40μM diclofenac, 50μM finasteride, 200μM flufenamic acid, 10μM Meloxican, 50μM Ebselen, and 20nM Flurbiprofen and 50μM. For the controls, cells were treated with an equal amount of DMSO or ethanol, which was less than 0.1% final concentration.

Apoptosis assays: Apoptosis was described before (11) and was assayed by using the Apoptotic Cell Death Detection ELISA (Roche) and/or the Cell Death Detection (Nuclear Matrix Protein) ELISA (Oncogene) according to the manufacturer's protocol.

Cell cycle analysis: Ovarian cancer cells were starverd and cultivated in serum free media. After 24 to 48 hours the cells were trypsinized, washed twice with cold PBS containing 2% fetal bovine serum and fixed in 70% Ethanol for 60mins at 4°C. The cells were then washed twice with PBS and stained with 200μl of propidium iodide stock solution (50μg/ml propidium iodide, 3.8mM Sodium trisphosphate in PBS) supplemented with 50μl RNase A (10μg/ml) for 3h at 4°C and then analysed with a FACScan cell sorter (Becton Dickinson). Ten thousand cells were collected and the cell cycle profiles were calculated by using the Cellquest Software.

Aim 2. Determine the best combination NSAIDs for induction of quiescent ovarian cancer apoptosis. (1-8)

Additionally, using the lowest dose of each NSAIDs (Figure 2), we have started to

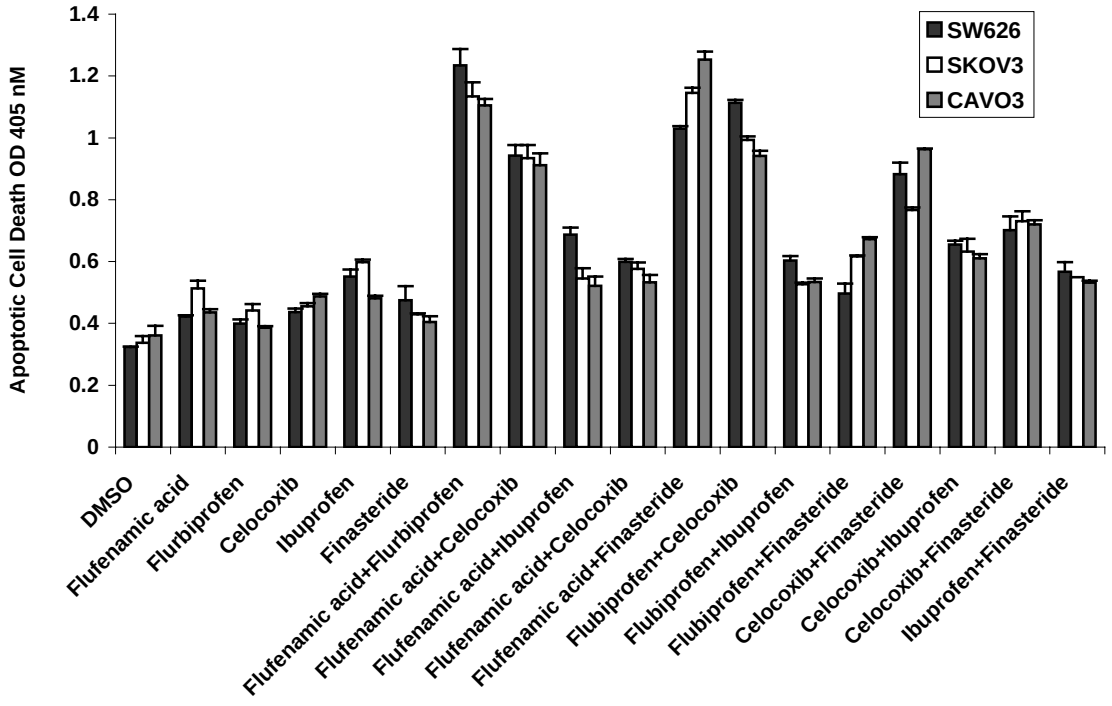

Figure 3. Multiple NSAIDs induce apoptosis in quiescent ovarian cancer cells. After synchronizing the cells in G0 stage by starvation method, SW626, CAOV3 and SKOV3 ovarian cancer cells were treated with 5μM Flufenamic acid, 4nM Flurbiprofen, 40μM Celocoxib, 25μM Finasteride or 200μM Ibuprofen and a combination of them or DMSO. Apoptosis was measured 24 hours after treatment and apoptosis were measured 24 hrs post-treatment. Data means ± s.d. of triplicate independent experiments for each treatment.

systematically analyze the apoptosis induction of a combination of NSAIDs in quiescent EOC cells. After synchronizing the cells at G0 stage, a panel of NSAIDs including Flufenamic acid, Flurbiprofen, Celocoxib, Finasteride and Ibuprofen were tested for their abilities to induce apoptosis alone and in combination thereof. The concentrations used here were the ones defined in Aim 1 as the lowest concentration able to induce apoptosis in quiescent EOC cells. SW626, CAOV3 and SKOV3 ovarian cancer cells were treated with 5μM Flufenamic acid, 4nM Flurbiprofen, 40μM Celocoxib, 25μM Finasteride or 200μM Ibuprofen and a combination of them. Apoptosis was measured 24 hours after treatment revealing that all combination of NSAIDs tested, induced apoptosis in ovarian cancer cells. However, some NSAIDs combinations had a stinking effect in the apoptosis induction. Strong inducers included Flufenamic acid+Flurbiprofen, Flufenamic acid+Finasteride, Flurbiprofen+Celocoxib and Flufenamic acid+Celocoxib (Figure 3)

Aim 3. Determine whether the combination of NSAIDs, which target quiescent cells, and NSAIDs with therapeutic effect in proliferating cells is more effective in inhibiting tumor formation or killing established ovarian cancer tumors in mice than mono-therapy (6-12 months).

To test whether NSAIDs is effective *in vivo* we use an EOC tumor model in SCID mice. We first evaluate whether NSAIDs such as Sulindac sulfide or a combination thereof will be able to prevent tumor formation EOC tumors in SCID mice. We first evaluated its toxicity in a pilot experiment. This first evaluation showed us the drugs develops adverse side effects if administer via IP injection. We then performed experiments using pumps, which releases the drugs in small concentration during 24 hours and added the drugs directly to the diets. This diminished the toxicity. Further, we also tested these compounds in combination in vivo. In parallel, we also started the experiments relating the effect of of NSAIDs using cells in the prolifetative stage. This experiment was aimed to define the efficacy of NSAIDs in vivo. We have already done all the in vitro experiments for NSAIDs in proliferative EOC cells as stated in our grant proposal applications. We then tested our most potent apoptosis inducer in the proliferative EOC cells, Sulindac sulfide, for inhibiting tumor formation in mice. 8-week-old female SCID-beige mice were were randomly divided into two groups and fed one of two diets through the entire experiment: AIN-93G as the control and the AIN-93G diet supplemented with 200ppm Sulindac sulfide and fed one of the experimental diets for 2 weeks. Mice were then inoculated subcutaneously with SKOV-3 or CAOV-3 EOC cells and continued on experimental diets. The experiment was finished soon and then when the average tumor weight in the control animals reaches 2–5% of the body weight. Sulindac sulfide Sulindac sulfide treatment reduced the average tumor weight by 50% (P-Value 0.0143) when compared to the control diet confirming its anti-tumor efficacy. We also tested testing in vivo our two of the most potent inducers of apoptosis in quiescent EOC cells, Flumenamic acid and Flurbiprofen.. 8-week-old female SCID-beige mice were were randomly divided into 3 groups and fed one of three diets through the entire experiment: AIN-93G as the control and the AIN-93G diet supplemented with 200ppm Flurbiprofen and AIN-93G diet supplemented with 300ppm Flufenamic acid and fed one of the experimental diets for 2 weeks. Mice were then inoculated subcutaneously with SKOV-3 or CAOV-3 EOC cells and continued on experimental diets. The experiment was finished soon and then when the average tumor weight in the control animals reaches 2–5% of the body weight. Flubirprofen and Flufenamic acid treatment reduced the average tumor weight by 42% and 24% respectively when compared to the control diet. We then, performed experiments with the best

inhibitor of quiescent EOC Flurbiprofen in combination with the best inhibitor of proliferative EOC, sulindac sulfide in vivo. The experimental methods were performed as described above. Sulindac Sulfide and Flurbiprofen treatment reduced the average tumor weight by 45% and 40%

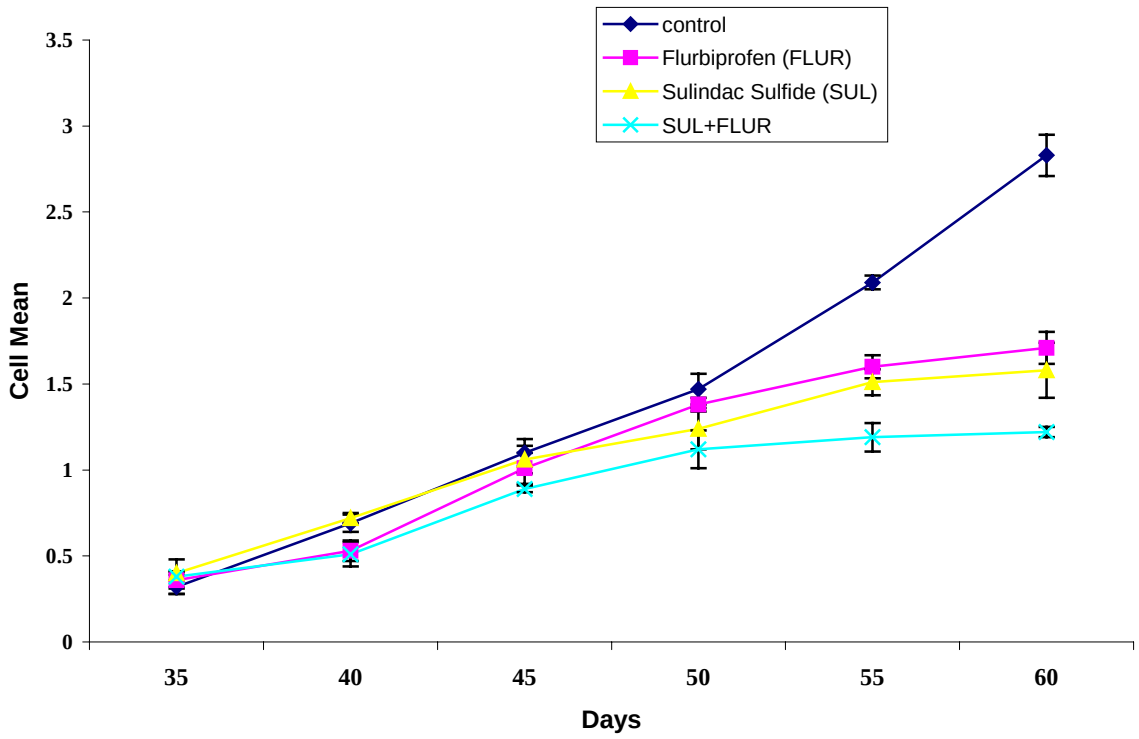

Figure 4. Combinatorial therapy with Sulindac sulfide and Flurbiprofen is more effective in inhibiting tumor formation than mono-therapy. For each inoculation, SKOV3 cells were injected subcutaneoslly into of SCID mice. The mice were randomly divided into 4 groups (n=8/group) and fed one of two diets through the entire experiment: AIN-93G as the control and the AIN-93G diet supplemented with 200ppm Sulindac sulfide, AIN-93G diet supplemented with 200ppm Flurbiprofen and a combination of both. The size of the tumors and tumor weight were measured 2 months after implantation

respectively when compared to the control diet. However, as seen in figure 4, the combination therapy (Flurbiprofen+Sulindac sulfide) had stronger effect regarding inhibiting tumor formation than the mono-therapies, reducing the tumor formation by 57% (P-Value 0.0135). We have previously demonstrate the imporatance of IL-24/MDA-7 genes and GADD45 family of genes in NSAIDs therapy in cancer. In order to identify the molecular mechanisms of NSAODs induction of apoptosis in quiescent EOC cells, we are currently studding the role of IL-24/MDA-7 genes and GADD45 family of genes.

Methods:

Animals, diets and implantation of SVOV-3 tumor cells. Eight-week-old female SCID-beige mice were purchased from Taconic (Germantown, NY), and housed in a pathogen-free environment. Immediately before implantation, SKOV-3 cells were trypsinized and resuspended in RPMI media. Cell viability was determined by trypan blue exclusion, and a single cell suspension with >90% viability was used for implantation. SKOV-3 cells ($2X10^6$ cells in 50µl)

were carefully injected subcutaneoslly via a 30-gauge needle as described previously (12). All procedures with animals were reviewed and approved by the Institutional Animal Care and Use Committee at the Beth Israel Deaconess Medical Center according to NIH guidelines. The mice were randomly divided into two groups (n=8/group) and fed one of two diets through the entire experiment: AIN-93G as the control and the AIN-93G diet supplemented with 200ppm Sulindac sulfide or Flurbiprofen or 300ppm Flufenamic acid. Body weight and food intake were measured weekly. At the end of the experiment (8 weeks), animals were sacrificed and tumors were carefully dissected and weighed.

Key research Accomplishments:

- We have successfully defined the strong NSAIDs inducers of apoptosis in quiescent ovarian cancer cells lines
- We have successfully determined the lowest dose of NSAIDs described above for induction of apoptosis in quiescent ovarian cancer cells lines
- We also have successfully determined the best NSAIDs combination for induction of apoptosis in quiescent ovarian cancer cells lines
- We also show that the efficacy of strong NSAIDs inducers of apoptosis in proliferative ovarian cancer cells lines in vivo
- Importantly, we have accomplished the main important goal of our grant. We successfully show that the efficacy of NSAIDs inducers of apoptosis in proliferative ovarian cancer in combination with the NSAIDs treatment for induction of apoptosis in quiescent ovarian cancer has stronger effect in vivo than the mono-therapy

Reportable Outcomes:
Work was presented in the AACR meeting San Diego.
Abstract number 1484, poster section 31, poster board 21
Combinatorial NSAID therapy in ovarian cancer: functional roles of MDA-7/IL-24 and GADD45 family genes. *Luiz F. Zerbini, Jason Cordeiro, Towia A. Libermann*. Harvard Institutes of Medicine, Boston, MA, BIDMC Genomics Center, Boston, MA

Conclusion: The lack of effective therapies for ovarian cancer reflects in part the lack of drug treatments that target quiescent tumor cells. Striking is the lack of knowledge about the mechanisms of action of drugs that may have the potential to target quiescent ovarian cancer cells. Here, we systematically and comprehensively decipher the effect of a whole panel of NSAIDs with potential anticancer activities and determine the potential best combination of complementary NSAIDs treatment for quiescent cells in ovarian cancer. Strong inducers included Flufenamic acid+Flurbiprofen, Flufenamic acid+Finasteride, Flurbiprofen+Celocoxib and Flufenamic acid+Celocoxib. Moreover, the best inducers of apoptosis in vitro, Flurbiprofen and flufenamic acid were also tested for the capacity of reducing tumor formation in vivo. Flurbiprofen proved to be the best NSAIDs treatment for quiescent EOC in vivo by reducing tumor formation by 42% while, flufenamic acid showed a modest reduction of 19%. Importantly, the combinatorial therapy of Sulindac sulfide (proliferative stage) and Flurbiprofen (quiescent stage) had a stronger effect in vivo compared with the mono-therapies. Furthermore, our in vivo

data allows us to rationally design a new combination therapy that could act more effectively against EOC. The use of NSAIDs or chemically modified NSAIDs which target specifically quiescent cancer cells in combination with other therapies that focus in the proliferative stage of the disease should enhance the apoptotic effect of NSAIDs on EOC.

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